Peptidomic study of casein proteolysis in bovine milk by *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331

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Abstract

Lactobacilli contain different cell envelope proteinases (CEPs) responsible for the hydrolysis of caseins and the release of various bioactive peptides. In this work, we explored the CEP activity of *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 whole cells towards β-, αS1-, κ- and αS2-caseins in bovine milk. Mass spectrometry analysis of fermented milk hydrolysates identified a total of 331 peptides, which were mainly derived from β-caseins (59.0 and 60.1% for PRA205 and PRA331, respectively). The analysis of αS1-casein (f1-23) cleavage site specificity congruently supports that *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 exhibited a mixed-type $CEP_{I/III}$ activity. PRA205 and PRA331 CEPs also showed cleavage site specificity toward β-casein, preferentially. These CEPs cleaved the peptide bond preferentially when hydrophobic or negatively charged amino acids were present. 13.5% and 13.7% of peptides released by *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 CEPs were found to have 100% homology with previously identified bioactive peptides.

1. Introduction

Food intake with the goal of improving human health is an ongoing focus for research. Recommendations for the consumption of certain nutritious fermented foods date back to the Hippocratic Corpus of Ancient Greece. The idea that lactic acid bacteria (LAB) fermenting milk are responsible for enhancing health and delaying the human aging was first proposed by the Russian scientist Elie Metchnikoff more than a century ago (Mackowiak, 2013). In the past years, research has documented a wide range of health benefits exerted by dairy LAB, especially immune and metabolic ones, and it is now focusing to decipher the microbial mechanisms underpinning these health-promoting effects (Reid, 2015).

Some beneficial effects exerted by LAB are due to the generation of secondary metabolites with health-promoting properties. The most important biogenic compounds in fermented milk are the bioactive peptides released from caseins via the LAB proteolytic system. Biological activities associated with such peptides include immunomodulatory, antibacterial, anti-hypertensive, antioxidant, mineral binding, and opioid-like properties (Brown et al., 2017). In addition, dairy LAB are auxotrophic for many amino acids and efficient casein breakdown is crucial to make LAB competitive as dairy starters (S-LAB), as well as suitable to survive in ripened cheeses as non-starter LAB (NS-LAB) (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). The amino acid release also contributes to the aroma compound formation during cheese ripening and impacts sensorial properties and consumer's acceptance of dairy foods (McSweeney & Sousa, 2000).

Cell envelope proteinases (CEPs) are large multi-domain proteins anchored to the cell wall that catalyse the first step of hydrolysis of milk caseins into peptides. Different transport systems then internalize these peptides into the cell, where they are further hydrolysed by numerous intracellular peptidases (Savijoki, Ingmer, & Varmanen, 2006). Six different types of CEPs have been described in several LAB species: PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Laloi, Atlan, Blanc, Gilbert, & Portalier, 1991); PrtH from *Lactobacillus helveticus* (Genay, Sadat, Gagnaire & Lortal, 2009); PrtL from *Lactobacillus delbrueckii* subsp. *lactis* (Villegas, Brown,

Savoy de Giori, & Hebert, 2015); PrtP from *Lactococcus lactis* (Kok, Leenhouts, Haandrikman, Ledeboer, & Venema, 1988), *Lactobacillus paracasei* (Holck & Naes, 1992), *Lactobacillus casei* (Fernández de Palencia, Peláez, Romero, & Martín-Hernández, 1997; Kojic, Fira, Banina, & Topisirovic, 1991), *Lactobacillus rhamnosus* (Guo et al., 2016) and *Lactobacillus plantarum* (Strahinic, Kojic, Tolinacki, Fira, & Topisirovic 2010); PrtR from *Lactobacillus rhamnosus* (Pastar et al., 2003); and PrtS from *Streptococcus thermophilus* (Siezen, 1999). These proteinases vary in substrate specificity, domain composition and cell wall anchoring, but all of them belong to the so-called subtilase family as they contain the catalytic serine protease domain showing sequence homology to the active site of subtilases (Savijoki et al., 2006; Sadat-Mekmene, Genay, Atlan, Lortal, & Gagnaire, 2011a). Most frequently, LAB possess only one CEP, but the presence of two CEPs has been described in lactobacilli (Sadat-Mekmene et al., 2011b).

Much of the current knowledge on LAB proteolytic system comes from studies on S-LAB species, such as *Lc. lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* and only few works has been done to elucidate the role of NS-LAB. Recently, the NS-LAB species *Lb. paracasei*, *Lb. casei* and *Lb. rhamnosus* were proven to generate bioactive casein-derived peptides during milk fermentation (Guo et al., 2016; Solieri, Rutella, & Tagliazucchi, 2015). *Lb. casei*/*Lb. paracasei* *PrtP*-encoded CEP was also demonstrated to degrade pro-inflammatory chemokines associated to inflammatory bowel diseases (Hormannsperger, von Schillde, & Haller, 2013). Consequently, there is an increasing interest to study NS-LAB proteases responsible for the release of bioactive peptides (Lozo et al., 2011).

In our previous work, we demonstrated that two mesophilic NS-LAB strains isolated from Parmigiano Reggiano ripened cheese, namely *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331, exhibit safety and technological performance compatible with probiotic properties (Solieri, Bianchi, Mottolese, Lemmetti, & Giudici, 2014). They also release the angiotensin-I-converting enzyme (ACE)-inhibiting peptides Valine-Proline-Proline (VPP) and Isoleucine-Proline-Proline (IPP) from caseins at doses that may exert antihypertensive effects *in vivo* (Solieri et al., 2015). Despite these

multiple interesting properties, the activity and specificity of CEPs from strains PRA205 and PRA331 remain unknown, as well as their potential to release additional milk-derived peptides other than VPP and IPP. The aim of this work was to fill this gap and to evaluate the pattern of casein breakdown by PRA205 and PRA331 whole cells CEP activities through a peptidomic approach.

2. Materials and Methods

2.1 Microorganisms, media and growth conditions

Lactobacillus casei PRA205 and *Lb. rhamnosus* PRA331 were isolated from ripened Parmigiano Reggiano cheese (Solieri, Bianchi, & Giudici, 2012) and deposited in Unimore Microbial Culture Collection (www.umcc.unimore.it) for long-term preservation. The cultures were activated from their frozen forms (stored in MRS medium supplemented with 25% (v/v) glycerol at −80°C) by transferring them in MRS broth and incubating at 37°C for 24h under anaerobic conditions. After two rounds of growth on the same medium, strains were routinely maintained on MRS medium supplemented with 7% (w/v) agar at 4°C for the duration of the experiments.

2.2 Inoculum preparation and milk fermentation

Milk fermentation trials were carried out in triplicate as follows. Single-colony cultures were inoculated in MRS broth for 24h at 37°C. Cells were washed twice with 50 mmol L^{-1} Tris-HCl buffer (pH 6.5), re-suspended in 10% (w/w) skimmed milk and used as pre-cultures (2% v/v) to inoculate milk batches prepared with 50 mL of ultra-high temperature-treated (UHT) skimmed bovine milk. Fermentation was carried out for 72h at 37°C at 10 rpm. pH values were determined over time as previously reported (Solieri et al., 2015). At the end of the fermentation (pH values ≤ 4.0 for at least two consecutive measurements), samples were taken to estimate milk protein hydrolysis, ACE-inhibitory and radical scavenging activities as reported in section 2.4.

2.3 Cell viability assay

PRA205 and PRA331 cells were harvested by centrifugation after 24, 48 and 72h of milk fermentation, twice washed with physiological solution (9 g L^{-1} NaCl) and re-suspended at the final concentration of 10^7 CFU mL^{-1}, according to the correlation curves between OD_{600nm} and CFU values previously established for every strain (Rutella, Tagliazucchi, & Solieri, 2016). Bacterial suspensions were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) and live/dead cell ratio was measured according to manufacture instructions. Fluorescence intensity was measured with a Jasco FP-6200 spectrofluorometer (Jasco, Orlando FL, U.S.A.).

2.4 Determination of milk protein hydrolysis, radical scavenging and angiotensin I-converting enzyme (ACE)-inhibitory activities

Milk protein hydrolysis and radical scavenging activity were determined on the TCA-soluble supernatants (peptidic fractions) obtained by treating fermented milk with 1% (w/v) TCA followed by a centrifugation at 10,000*g* for 20 min (4°C). In particular, milk protein hydrolysis was determined by measuring the amounts of released amino groups using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay (Adler-Nissen, 1979). Briefly, 50 µL of appropriately diluted peptidic fractions were mixed with 400 µL of sodium phosphate buffer (0.1 mmol L^{-1}; pH 8.2) and 400 µL of 0.1% TNBS solution (prepared in the same sodium phosphate buffer). After 60 min of incubation at 50°C, the reactions were stopped by adding 800 µL of HCl 0.1 mmol L^{-1}. The absorbance values at 340 nm were read using a Jasco V-550 UV/Vis spectrophotometer (Jasco, Orlando, FL, USA.). A calibration curve was prepared using leucine as standard (range 0.1-2.0 mmol L^{-1}) and the results were expressed as mmol L-1 of leucine equivalents.

The antioxidant activity of the peptidic fractions was measured as radical scavenging activity using the ABTS radical cation decolourization assay (Re et al., 1999) and expressed as mg L^{-1} of Trolox.

ACE-inhibitory (ACEi) activity was determined according to Ronca-Testoni (1983) on the ultra-filtrated fraction obtained from fermented milk as previously reported (Solieri et al., 2015). The tripeptide 2-furanacryloyl–phenylalanylglycylglycine (FAPGG) was used as substrate assay and the ACEi activity was calculated as percent of inhibition (ACEi%).

Three analytical replicates were run for each sample collected at the end of each fermentation trial (carried out in triplicate) in all the assays.

2.5 Determination of peptides with nanoflow LC-ESI-QTOF MS analysis

Peptidomic analysis was performed by injecting the TCA-soluble supernatant of fermented milk on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass QTOF LC/MS via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA, USA) as described in Tagliazucchi, Helal, Verzelloni, Bellesia, & Conte (2016). Chromatographic separation was performed on a ProtID-Chip-43(II) including a 4 mm 40 nL enrichment column and a 43 mm × 75 μm analytical column, both packed with a Zorbax 300SB 5 μm C18 phase (Agilent Technologies). The mobile phase consisted of (A) H_2O/acetonitrile/formic acid (96.9:3:0.1, v/v/v) and (B) acetonitrile/H_2O/formic acid (94.9:5:0.1, v/v/v). The sample (2 μL) was loaded onto the Chip enrichment column at a flow rate of 4 μL min^{-1} with a mobile phase consisting of 100% A using a G1376A capillary pump. A flush volume of 2 μL and a flush-out factor of 5 were used. After valve switching, a gradient elution was performed throughout the enrichment and analytical columns at 500 nL min^{-1} using a G2226A nano pump. The gradient started at 0% B for 1 min, and then linearly ramped up to 90% B in 70 min. The mobile phase composition was maintained at 90% B for 15 min in order to wash both enrichment and analytical columns. The mass spectrometer was tuned and calibrated according to the manufacturer's instructions in extended dynamic range (2 GHz) mode as reported by Dei Più et al. (2014).

For peptide identification, MS/MS spectra were converted to .mgf files and were then searched against the Swiss-Prot database using Protein Prospector (http://prospector.ucsf.edu) and

MASCOT (Matrix Science, Boston, MA, USA) protein identification softwares. The following parameters were considered: enzyme, none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal number of post-translational modifications permitted in a single peptide, 4. We considered only peptides with a best expected value lower than 0.05 that corresponded to $P<0.01$. The assignment process was complemented and validated by the manual inspection of MS/MS spectra. Three replicates for each fermentation trial was injected in the mass spectrometer and only the peptides present in at least two replicates were considered significant and included in the analysis.

2.6 Identification of bioactive peptides

The identified peptides in milk samples were investigated for literature-identified bioactive peptides using the BIOPEP database and the Milk Bioactive Peptide Database (MBPDB) (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008; Nielsen, Beverly, Qu, & Dallas, 2017). Only peptides with 100% homology to known functional peptides were considered as bioactive peptides.

The relative amount of the bioactive peptides was estimated by integrating the area under the peak (AUP). AUP was measured from the extracted ion chromatograms (EIC) obtained for each peptide and normalized to the peptide content of milk hydrolysates. The peptide content was determined at the end of the fermentation trials by using the TNBS method as described in section 2.4 and expressing the results as mg of leucine equivalent mL^{-1}

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2.7 Calculation of the cleavage specificity

The cleavage probability and positive or negative influence on the cleavage of an amino acid in the P1 and P1’ subsites were calculated according to Keyl (1992).

The subsite nomenclature was according to Schechter & Berger (1967) where the amino acid residues are designated as P1 in the N-terminal direction (on left of the sequence) and Pl’ in the C-terminal direction (on right of the sequence) from the cleaved bond. The subsite P1 interacts with

the subsite S1 in the enzyme active site, whereas the subsite P1' interact with the subsite S1' in the enzyme active site. Therefore, the peptidic bond cleaved by the protease was defined as the P1-P1' bond. We quantitatively analysed the influence of specific amino acid residues in position P1 or P1'on the CEP cleavage probability.

If the amino acid residue *A* is in the position *n* (P1 or P1' subsite), the cleavage probability of the P1–P1' bond will be:

$$\%Pn = \frac{total\ amino\ acid\ A\ cleaved\ in\ position\ n}{total\ amino\ acid\ A\ in\ proteins} \times 100$$

and in consequence the mean cleavage probability:

$$\%\overline{Pn} = \sum_{\#=1}^{20} \frac{\%Pn}{20}$$

The coefficient *Kn* was used to quantify the positive or negative influence of an amino acid residue *A* in the P1 and P1' subsites:

$$Kn = \frac{\%Pn}{\%\overline{Pn}} - 1$$

Kn values >0 indicated a positive influence of the amino acid *A* in the specific subsite on the cleavage of the P1-P1' bond, whereas *Kn* values <0 suggested a negative effect on the cleavage.

2.8 Statistical analysis

All data are presented as mean ± standard deviation (SD) for three replicates. The Student's t-test was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered significant with $P < 0.05$. Venn diagrams were drawn using the online tool VENNY 2.1.0 (Oliveros, 2015).

3. Results and Discussion

3.1 Characterization of fermented milk

Analysis of CEP activities on purified caseins could tend to overestimate the true caseinolytic capability of whole cells towards casein micelles in milk (Sadat-Mekmen et al., 2011b). The use of purified CEPs instead whole-cell anchored CEPs may also modify the specificity of the proteinase towards caseins (Fernández de Palencia et al., 1997). Therefore, we decided to evaluate the CEP activities of whole cells of *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 towards the caseins in UHT milk.

Milk samples were inoculated with standardized amounts of PRA205 and PRA331 single cultures without any pre-adaptation step. After 72h of incubation, pH values were 4.00 in both sets of samples and remained stable over time. At the end of fermentation, strain PRA205 showed value of leucine equivalents of 10.93 ± 0.93 mmol L^{-1}, whereas PRA331 of 6.40 ± 0.92 mmol L^{-1}. These data agree with earlier results showing that PRA205 is more proteolytic than PRA331 towards milk caseins (Solieri et al., 2015). *Lb. casei* PRA205 produced milk hydrolysates with ACEi activity higher than that exhibited by milk hydrolysates with *Lb. rhamnosus* PRA331 (75.8 ± 3.2 *vs* 68.5 ± 2.6 ACEi%). Similarly, antioxidant activity was slightly higher in hydrolysates by strain PRA205 than the hydrolysates by strain PRA331 (249.12 ± 15.10 *vs* 202.57 ± 18.66 mg L^{-1} of trolox, respectively).

The level of bacterial lysis during milk fermentation was monitored to exclude that the peptides could be generated by intracellular peptidases released into the hydrolysates. Cell viability was estimated app. 100% for both PRA205 and PRA331 after 24 and 48h of incubation (data not showed). Interestingly, at the end of milk fermentation the percentage of viable cells was 90.49 ± 0.74% and 94.59 ± 5.70% for PRA205 and PRA331, respectively. These data indicated that no significant lysis occurred during milk fermentation and supported that the observed casein

proteolysis was mainly due to the action of CEPs anchored on the whole cells rather than intracellular proteinases or peptidases.

3.2. Peptidomic analysis of milk fermented with Lactobacillus casei PRA205 and Lactobacillus rhamnosus PRA331

Mass spectrometry analysis was used to identify the full set of peptides present in milk hydrolysates by the selected strains. A total of 331 milk peptides were released by the CEPs activities of PRA205 and PRA331 whole cells. In particular, 178 peptides were identified in PRA205 samples (see supplementary online **Tables S1-S4** and **Figures S1-S4**) and 153 peptides in PRA331 samples (see supplementary online **Tables S5-S8** and **Figures S1-S4**).

The analysis of the identified sequences according to their protein of origin showed that the main identified peptides were derived from β-casein, which was the preferred substrate over αS1-, κ- and αS2-caseins. The β-casein-derived peptides were 59.0 and 60.1% of the total identified peptides in PRA205 and PRA331 samples, respectively, followed by αS1-casein-derived peptides (18.5 and 19.0% of the total identified peptides in PRA205 and PRA331 samples, respectively) and κ-casein-derived peptides (16.3 and 15.0% of the total identified peptides in PRA205 and PRA331 samples, respectively). PRA205 and PRA331 CEPs poorly hydrolysed αS2-casein, resulting in only 11 (corresponding to the 6.2% of total identified peptides) and 8 peptides (corresponding to the 5.9% of total identified peptides), respectively. As expected, no significant proteolysis of whey proteins was observed for both the strains. The Venn diagram (**Figure 1**) showed that 24.6 and 14.0% of peptides were specific for PRA205 and PRA331 milk hydrolysates, respectively. The majority of the identified peptides were found in both the milk hydrolysates, suggesting that PRA205 and PRA331 share a similar caseinolytic pattern.

CEPs are classified on the basis on their caseinolytic specificity (Kunji et al. 1996). Typically, two CEPs have been identified: a P_I-type, which preferentially hydrolyses β-casein, and a P_{III}-type, which acts on αS1-, β- and κ –caseins equally well (Pritchard & Coolbear, 1993; Visser,

Exterkate, Slangen & de Veer 1986). A third group, termed P_I/P_{III}-type has been described to classify intermediate proteases, capable to cleave β-casein like the P_I-type and, to a lesser extent, α- and κ-caseins (Exterkate, Alting, & Bruinenberg, 1993). Both *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 exhibited a predominant CEP activity towards β-casein, and a lower proteolytic activity towards α- and κ-caseins. Cell viability data allowed us to exclude that intracellular aminopeptidase released by lysed cells may significantly contribute to this pattern of casein breakdown. Furthermore, no extracellular aminopeptidases have been reported for *Lb. casei* and *Lb. rhamnosus* (Christensen, Dudley, Pederson, & Steele, 1999). Overall, these evidences support that the observed CEP activities could be due to the mixed P_I/P_{III}-type proteases. P_I/P_{III}-type proteases have been characterized in lactobacilli (Fernandez de Palencia et al., 1997; Sadat-Mekmene et al., 2011a Villegas et al., 2015) and lactococci (Nikolić, Tolinački, Fira, Golić, & Topisirović, 2009). In particular, like PRA331, *Lb. rhamnosus* BGT10 has PrtR protease suitable to cleave both β- and α-caseins.

3.3. Analysis of the αS1-casein (f1-23) cleavage sites

CEPs are commonly classified according to their specificities toward the αS1-casein fragment comprising residues from 1 to 23 (Exterkate, 1995). In strains PRA205 and PRA331, CEPs hydrolysed the αS1-casein (f1-23) fragment at the H_8-Q_9, Q_9-G_{10}, Q_{13}-E_{14}, N_{17}-E_{18} and L_{21}-R_{22} positions, respectively (**Figure 2**). In addition, PRA331 also cleaved at the L_{16}-N_{17} position. The majority of these cleavage sites are typical of mixed P_I/P_{III}-type CEPs isolated from several lactococci, *S. thermophilus* CNRZ 385, *Lb. delbrueckii* subsp. *lactis* CRL 581 and *Lb. helveticus* L89 (Exterkate, 1995; Fernandez-Espla, Garault, Monnet, & Rul, 2000; Hebert et al., 2008; Kunji et al., 1996). Two additional cleavage sites were found at the P_2-K_3 and E_{18}-N_{19} positions. The cleavage site E_{18}-N_{19} has been already reported for the CEP of *Lb. delbrueckii* subsp. *lactis* CRL 581 (Hebert et al., 2008), whereas the cleavage site P_2-K_3 has never been identified in any CEPs previously described from lactobacilli. These results collectively suggested that CEPs from *Lb.*

casei PRA205 and *Lb. rhamnosus* PRA331 could belong to the mixed P_I/P_{III}-type. This result disagrees with the P_I-type CEP previously characterized in *Lb. casei* HN14 (Kojic et al., 1991), while it is consistent with the mixed P_I/P_{III} type CEPs isolated from *Lb. rhamnosus* CGMCC11055 and *Lb. casei* subsp. *casei* IFLP 731 (Guo et al., 2016; Fernández de Palencia et al., 1997). Overall, these evidences strongly support the high level of intra- and inter-species variability in protease repertoire exhibited by lactobacilli (Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010). As reported above, the cell viability near to 100% measured at the end of the fermentation trials allowed us to exclude that intracellular peptidase released from lysed cells may contribute to the hydrolysis of the fragment αS1-casein (f1-23). Indeed, as reported by Christensen, Broadbent, & Steele (2003), the presence of cytoplasmic peptidase should results in an almost complete breakdown of the peptide αS1-casein (f1-9).

3.4. Analysis of the β-casein cleavage site-specificity

The cleavage site-specificity of PRA205 and PRA331 CEPs was determined using β-casein as preferred substrate (**Figure 3**). In total, 76 and 72 different cleavage sites were detected in samples hydrolysed by PRA205 and PRA331 whole cells, respectively. They constitute 36.5 and 34.6% of all peptide bonds present in β-casein, showing that *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 CEPs have a very broad substrate specificity. These CEPs have almost the same specificity, as they shared 65% of cleavage sites.

Amino acid sequence analysis of the identified peptides revealed that the cleavage sites were not concentrated at the N- or C-terminus, but rather distributed throughout the entire β-casein sequence for both the CEPs activities (**Figure 3**). Most of the proteinases previously described in lactobacilli have been proven to preferentially hydrolyse the C-terminal of β-casein (Lozo et al., 2011). Recently, the PrtP proteinase isolated from *Lb. rhamnosus* CGMCC11055 breakdowns sites distributed along the whole β-casein sequence, like PRA205 and PRA331 CEPs (Guo et al., 2016).

Furthermore, we calculated the cleavage probability (%*Pn*) of the *Lb. casei* PRA205 CEP at the P1 and P1' positions **(Table 1)**. This CEP cleaved preferentially when the P1 position was occupied by the hydrophobic amino acids M, L and F or the negatively charged amino acids Q and N primarily, and by the polar un-charged amino acid E to a lesser extent. **Table 1** also shows how the amino acids at the P1' position affected cleavage occurrence. PRA205 CEP exhibited cleavage preference towards the residues S, N, A and H in this position, whereas had a reduced preference for M, D, R and Y. Coefficients *Kn* were calculated to quantify the influence of different amino acid residues on the P1-P1'cleavage probabilities (**Figure 4**). Amino acids N, M, Q, F and L in the P1 position and amino acids S, N, A and H in the P1' position exerted the strongest positive effects on cleavage occurrence. The amino acids E in P1 position and V, M, D, R and Y in P1' position also exerted a positive but weaker effect on cleavage probability. By contrast, G, I, P and D in the P1 position and P and I in the P1' position strongly inhibited the cleavage probability. Similarly, a negative effect was also found for the amino acids E and T at the P1' position. Previous works found that CEPs preferentially cleave negatively charged and hydrophobic amino acids (Hebert et al., 2008; Juillard et al., 1995; Lozo et al., 2011; Monnet, Ley, & Gonzalez, 1992). For instance, Q and E at the P1 position positively affect the cleavage by CEP from *Lb. delbrueckii* subsp. *lactis* CRL 581 (Hebert et al., 2008), whereas the occurrence of Q and F at the same position positively affects the cleavage by CEPs from *Lb. rhamnosus* BGT10, *Lb. helveticus* BGRA43 and *Lb. paracasei* subsp. *paracasei* BGHN14 (Lozo et al., 2011). In *Lb. casei* PRA205 CEP exhibited a pattern of cleavage site preferences similar to P_I/P_{III}-type CEP described in *Lc. lactis* subsp. *lactis* strain NCDO763 (Monnet, Ley, & Gonzalez, 1992), but different from those described for the P_I-type CEP in *Lc. lactis* subsp. *cremoris* strain Wg2 and for the P_I/P_{III}-type CEP in *Lb. rhamnosus* strain CGMCC11055. In strain Wg2 the residue Y at the P1 position and the residues N and T at the P1' position were preferred (Juillard et al., 1995), whereas in strain CGMCC11055 the residue P was preferred in both P1 and P1' subsites (Guo et al., 2016). By contrast, strain NCDO763 had a CEP activity positively affected by Q and N at the P1 position, and by S and A at the P1' position

(Monnet et al., 1992). Additionally, the residue P in both the P1 and P1' positons negatively affected CEP cleavage in NCDO763 (Monnet et al., 1992). Similarly, the presence of a P residue bound to one of the preferred cleaved amino acids prevented CEP from *Lb. casei* PRA205 to cut the peptidic bond. For example, the preferentially cleaved amino acids Q and L formed seven and nine peptidic bonds with the amino acid P, respectively, but no one of these bonds was cleaved by PRA205 CEP (**Figure 3**). Finally, PRA205 CEP activity displayed the following two unique properties: M at the P1 position exerted a strong positive effect on cleavage occurrence, whereas I in both the P1 and P1' positions exerted a strong negative effect. To the best of our knowledge, this cleavage site-specificity pattern has never been described in lactobacilli.

As reported in **Table 1** and **Figure 4**, CEP from *Lb. rhamnosus* PRA331 had a profile of cleavage specificity similar to *Lb. casei* PRA205. The main differences were the negative effect exerted by the amino acid H at the P1 position and the lack of the positive effect exerted by A at the P1' position.

3.5. Identification of bioactive peptides using functional peptides databases

The peptides cleaved by PRA205 and PRA331 CEPs in milk hydrolysates were searched against the general bioactive peptide database BIOPEP (Minkiewicz et al., 2008) and the milk bioactive peptide database MBPDB (Nielsen et al., 2017), in order to find peptides which match sequences to known bioactive peptides. Out of 331 identified peptides, 24 shared 100% homologies with functional peptides previously reported to have various bioactivities (**Table 2**). These bioactive peptides represented 13.5% and 13.7% of the peptides totally released by *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 whole cells, respectively. Twenty-one peptides were commonly released by both the strains, whereas three peptides were uniquely identified in samples hydrolysed by *Lb. casei* PRA205 (**Figure 1**). Nineteen bioactive peptides derived from β-casein, four from αS1-casein and one from αS2-casein, whereas no bioactive peptides were found from κ-casein. (**Table 2**). The three *Lb. casei* PRA205-specific bioactive peptides were the β-casein fragments 192-209

(LYQEPVLGPVRGPFPIIV), 58-72 (LVYPFPGPIPNSLPQ) and 8-14 (VPGEIVE). Eighteen peptides were ACE-inhibitors, two had immunomodulatory activity, one showed dipeptidyl-peptidase IV (DPPIV) inhibitory activity and one was an antimicrobial peptide. Two peptides, VYPFPGPIPN and YPFPGPIPN, were multi-functional bioactive peptides with ACEi, antioxidant and opioid agonist or ACEi, DPPIV-inhibitory and opioid agonist activities, respectively (**Table 2**). Among the peptides with ACEi activity, YPFPGPIPN, KVLPVPQ, RPKHPIKHQ and LHLPLP showed *in vivo* antihypertensive activity in spontaneously hypertensive rat (Maeno, Yamamoto, & Takano, 1996; Quirós et al., 2007; Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000). For all the other identified bioactive peptides, the bioactivity was previously demonstrated with *in vitro* assays.

The physiological effects of bioactive peptides depend on their capability to arrive at the target organs in an active form (Udenigwe, & Fogliano, 2017). This required resistance to gastrointestinal proteases and brush border membrane peptidases, and absorption through the intestinal epithelium. Usually, P-containing peptides are considered resistant to degradation by digestive proteases. Peptides containing from one to four P residues in their sequences and with, in many cases, P at or near to carboxylic end, were found to survive *in vitro* gastro-intestinal digestion (Tagliazucchi et al., 2016). Among the identified bioactive peptides, seven of them were able to survive *in vitro* gastro-intestinal digestion (Picariello et al., 2015; Tagliazucchi et al., 2016) and were also found in human gastro-intestinal tract (Boutrou et al., 2013), namely DKIHPF, VYPFPGPIPN, YPFPGPIPN, NIPPLTQTPV, LHLPLP, FVAPFPEVF and VAPFPEVF. The intestinal brush-border membrane and the colonic cells also contain aminopeptidases e specific prolyl peptidases. However, the great quantity of P-rich peptides and the presence of peptides with inhibitory activities (as for example against DPP-IV and intestinal ACE) may slow down the action of prolyl peptidases, protecting the short peptides from hydrolysis and favouring their biological actions. Short peptides (two or three amino acids) are absorbed intact across the brush border membrane by a specific peptide transport system, whereas largest peptides via paracellular and/or

transcellular mechanisms (Vermeirssen, Van Camp, & Verstraete, 2004). The peptide LHLPLP showed *in vivo* anti-hypertensive activity in rats, and, after incubation with Caco-2 cells, it was hydrolysed by cellular peptidases to HLPLP prior to transport across the intestinal epithelium (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008). The penta-peptide HLPLP showed an absolute bioavailability of 5.2% and an absorption half-life of 2.8 min in rats (Sánchez-Rivera et al., 2014). HLPLP was found to be hydrolysed by plasma peptidases in shorter peptides, which retained the anti-hypertensive properties in rats (Quirós et al., 2008; Sánchez-Rivera et al., 2014; Sánchez-Rivera et al., 2016). No data on absorption or pharmacokinetics of the other identified bioactive peptides are available in literature.

3.6. Bioactive peptides abundance across PRA205 and PRA331 fermented milk

Each identified bioactive peptide was relatively quantified in the samples by integrating the area under the peak (AUP) from the extracted ion chromatogram. The ionization of specific peptides in mass spectrometry experiments is a major limitation in quantitative analysis with electrospray ionization mass spectrometry (ESI-MS). The relative ionization of individual peptides is dependent on intrinsic and extrinsic factors. The most important extrinsic factor is the so-called "matrix effect" which is caused by the co-elution of matrix components (typically salts, ions, highly polar compounds and carbohydrates) that alter, either suppressing or enhancing, the ionization of the target analyte (Furey, Moriarty, Bane, Kinsella, & Lehane 2013). The intrinsic factor are related to the amino acid sequence of the peptides. Some amino acids, such as basic or hydrophobic amino acids, are ionized more efficiently than the others and gave more intense signal in ESI-MS experiments (Cech, & Enke, 2000). Here, we compared the relative amount (expressed as AUP) of the same peptide in two different hydrolysates coming from the same matrix (fermented milk), thus we can exclude errors related to the extrinsic effect and assume that differences in peak intensity of the same analyte accurately reflects relative differences in its abundance.

Among the bioactive peptides detected in both milk hydrolysates, 17 exhibited mean abundances significantly different between *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 ($P<0.05$). In particular, five peptides were more abundant in *Lb. casei* PRA205 milk hydrolysates and twelve in *Lb. rhamnosus* PRA331 milk hydrolysates (**Table 2**). When peptides intensities were summed, the bioactive peptides released by *Lb. rhamnosus* PRA331 whole cells were significantly higher than those released by *Lb. casei* PRA205 ($11.50 \times 10^{10} \pm 0.39 \times 10^{10}$ vs. $8.63 \times 10^{10} \pm 0.37 \times 10^{10}$; $P=0.0007$).

4. Conclusions

Nowadays, there is an increasing interest in developing novel dairy healthy products. Studies on strains *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 may represent a proof-of-concept of the working flowchart to develop novel functional adjunct culture and the subsequent functional delivery food. We isolated proteolytic and stress-resistant strains from a stressful food niche (no sugars available, high salt concentration, low aw), such as Parmigiano Reggiano, and identified them using a rigorous polyphasic identification frame-shift (Solieri et al., 2012). We demonstrated that all of them are safe (sensitive to all tested antibiotics) and some resistant to *in vitro* gastro-intestinal conditions (Solieri et al., 2014). We positively tested their ability to release VPP and IPP both in milk (Solieri et al., 2015) and in yogurt (Rutella et al., 2016), supporting the development of a double functional food, i.e. yogurt enriched in potentially probiotic viable cells and in antihypertensive peptides released by themselves. In this work, we characterized the CEPs that are the first enzymatic activities responsible for these relevant proteolytic features. For this purpose, a cutting-edge peptidomic approach was implemented in order to define the pattern of caseins breakdown by CEP activity from whole cells grown in milk. We demonstrated that CEPs activities of *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331showed two unique features: a new cleavage site (P_2-K_3) on the αS1-casein fragment 1-23 and a novel pattern of β-casein cleavage site-specificity. Through a BIOPEP and MBPDB databases analysis, we also demonstrated that several identified

peptides matched the sequences of previously reported bioactive peptides. This information could be relevant, mainly considering the wide heterogeneity in distribution of different proteinase-encoding genes among and within *Lactobacillus* species. Comparative genome analysis showed that lactobacilli strongly differ in the components of their proteolytic systems at strain level (Liu et al., 2010). This strain-specificity accounts for the high phenotypic diversity in caseinolytic activity and in of the resulting released bioactive peptides, as well as makes necessary to deeply characterize each strain selected for functional food applications. However, it is important to note that protein hydrolysis catalysed by proteases is a dynamic process and that, in the present work, bioactive peptides were identified in fermented milk samples at one single time. We cannot exclude that shorter or longer incubation of milk with *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 whole cells may result in a different bioactive peptide profile of the samples. In addition, future biochemical assays with the synthesized peptides are needed to complement the *in silico* evidences collected here.

Overall, the results provided in the present work will increase the knowledge about the proteolytic system of two important NS-LAB species, such as *Lb. casei* and *Lb. rhamnosus*, which are poorly studied compared to the best-described lactococci and thermophilic lactobacilli. Finally, since strains PRA205 and PRA331 released several potential bioactive peptides, they could be promising functional starters or adjunct cultures for formulating dairy products with health properties.

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Figure Captions

Figure 1. Venn diagram showing differences between *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 CEPs in patterns of peptides and bioactive peptides cleaved from milk caseins. The complete pattern of peptides identified at the end of the fermentation trials by mass spectrometry can be found in Supplementary on line **Tables S1-S8**. In the preparation of the Venn diagram related to bioactive peptides, only peptides found from the literature to have 100% homology to known functional peptides were reported in the **Figure**. Peptides present in at least two of a triplicate's samples were considered present.

Figure 2. **Specificity of CEPs from strains *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 toward αS1-casein fragment 1-23**. The cleavage sites are indicated by arrows.

Figure 3. Distribution of the cleavage sites identified in the primary sequences of β-casein by CEPs from *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The cleavage sites are indicated by arrows.

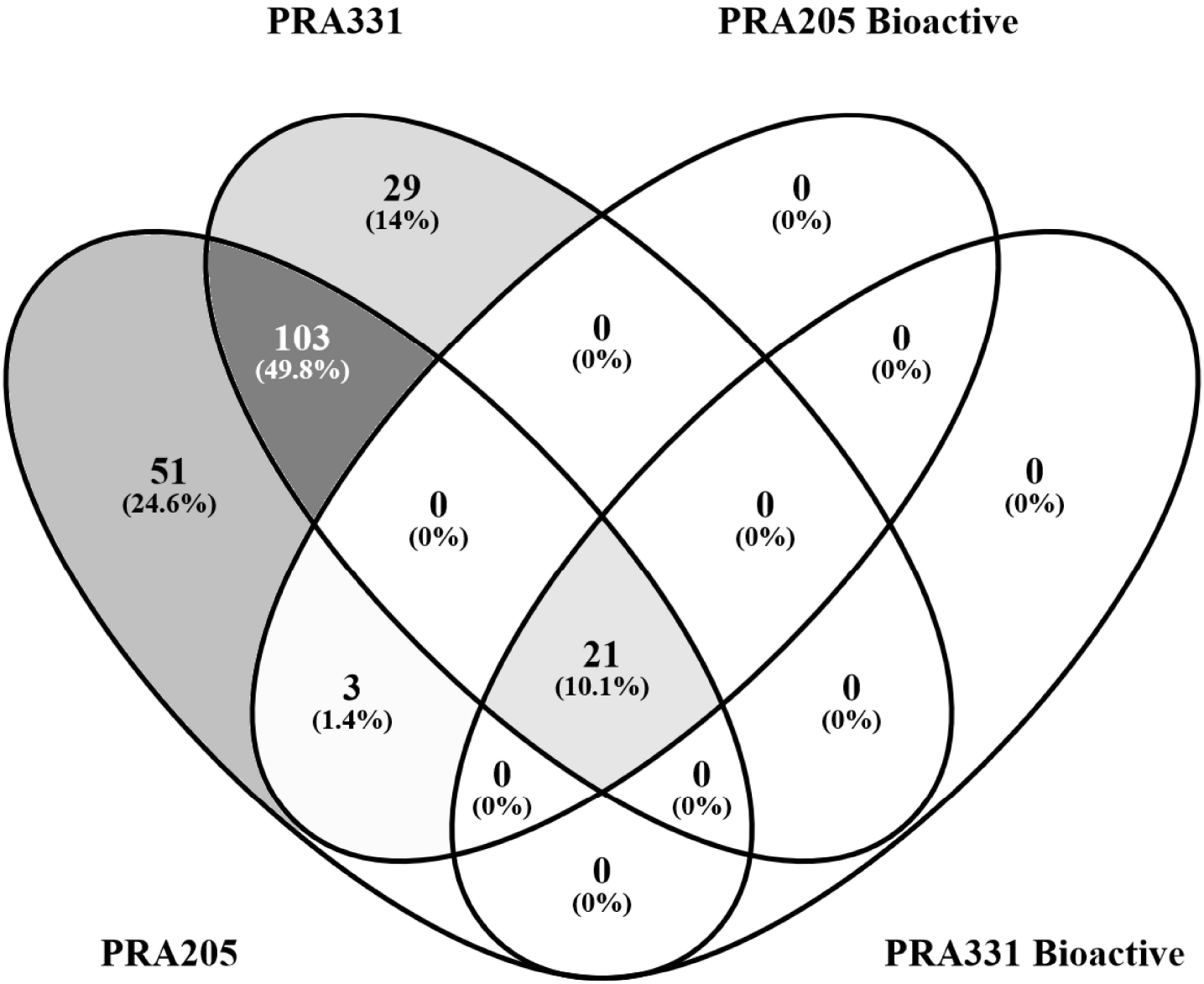
Figure 4. **Cleavage preference of *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 CEPs towards eighteen amino acids at the P1 and P1' subsites.** (**A**) Influence of the different amino acids in the P1 subsite by CEP from *Lb. casei* PRA205. (**B**) Influence of the different amino acids in the P1' subsite by CEP from *Lb. casei* PRA205. (**C**) Influence of the different amino acids in the P1 subsite by CEP from *Lb. rhamnosus* PRA331. (**D**) Influence of the different amino acids in the P1' subsite by CEP from *Lb. rhamnosus* PRA331. See materials and methods section for the calculation of the coefficient *Kn*. Positive and negative values indicate a positive or negative influence exerted by each residue on the cleavage of the P1-P1' bond, respectively. Please note that the amino acid C is not present in the sequence of β-casein, whereas W was omitted from the analysis since it occurs once in the β-casein sequence.

Table 3. Peptides with previously demonstrated bioactivity identified in the milk hydrolysates by whole cells of *Lactobacillus casei* PRA205 or *Lactobacillus rhamnosus* PRA331.

Sequence	*Fragment*	*Bioactivity*	*PRA205 relative amount[a] (±SD)*	*PRA331 relative amount[a] (±SD)*	*P-value*	*Reference*
LNVPGEIVE	β-casein f(6-14)	ACEi	$1.13x10^9 \pm 1.26x10^8$	$1.30x10^9 \pm 4.21x10^7$	0.0876	Gobbetti et al., 2000
VPGEIVE	β-casein f(8-14)	DPPIV-inhibitor	$4.30x10^8 \pm 3.38x10^7$	n.d.	/	Nongonierma et al., 2016
DKIHPF	β-casein f(47-52)	ACEi	$2.37x10^{10} \pm 3.37x10^9$	$2.36x10^{10} \pm 6.25x10^8$	0.4925	Gobbetti et al., 2000
LVYPFPGPIPNSLPQ	β-casein f(58-72)	ACE-inhibitor	$3.44x10^8 \pm 3.89x10^7$	n.d.	/	Smacchi et al., 2008
VYPFPGPIPN	β-casein f(59-68)	ACEi Antioxidant Opioid agonist	$8.19x10^9 \pm 7.04x10^8$	$1.62x10^{10} \pm 4.17x10^8$	0.0004	Eisele et al., 2013
YPFPGPIPN	β-casein f(60-68)	ACEi DPPIV-inhibitor Opioid agonist	$3.34x10^9 \pm 1.30x10^8$	$1.03x10^{10} \pm 1.89x10^9$	0.0030	Saito et al., 2000
NIPPLTQTPV	β-casein f(73-82)	ACEi	$9.51x10^9 \pm 5.72x10^8$	$5.96x10^9 \pm 4.64x10^8$	0.0027	Gobbetti et al., 2000
NLHLPLP	β-casein f(132-138)	ACEi	$1.94x10^9 \pm 2.09x10^8$	$7.81x10^8 \pm 1.22x10^8$	0.0031	Kohmura et al., 1989
NLHLPLPLL	β-casein f(132-140)	ACEi	$8.78x10^8 \pm 3.33x10^7$	$5.92x10^8 \pm 1.39x10^7$	0.0037	Robert et al., 2004
LHLPLP	β-casein f(133-138)	ACEi	$3.40x10^8 \pm 3.47x10^7$	$7.64x10^8 \pm 6.41x10^7$	0.0072	Kohmura et al., 1989
LHLPLPL	β-casein f(133-139)	ACEi	$2.55x10^9 \pm 2.85x10^8$	$3.06x10^9 \pm 2.19x10^8$	0.0920	Quiros et al., 2007
SQSKVLPVPQ	β-casein f(166-175)	ACEi	$3.49x10^8 \pm 3.51x10^7$	$6.14x10^8 \pm 7.03x10^7$	0.0050	Hayes et al., 2007
SKVLPVPQ	β-casein f(168-175)	ACEi	$8.32x10^8 \pm 1.07x10^8$	$1.07x10^9 \pm 7.70x10^7$	0.0380	Yamamoto et al., 1994
KVLPVPQ	β-casein f(169-175)	ACEi	$6.05x10^9 \pm 5.74x10^8$	$1.40x10^{10} \pm 1.76x10^9$	0.0022	Maeno et al., 1996
RDMPIQAF	β-casein f(183-190)	ACEi	$7.64x10^9 \pm 2.57x10^8$	$1.33x10^9 \pm 8.06x10^7$	<0.0001	Yamamoto et al., 1994
LYQEPVLGPVRGPFPIIV	β-casein f(192-209)	Immunomodulator	$3.19x10^8 \pm 4.13x10^7$	n.d.		Boutrou et al., 2013
YQEPVLGPVRGPFPIIV	β-casein f(193-209)	Immunomodulator	$1.69x10^9 \pm 3.86x10^8$	$1.21x10^9 \pm 1.57x10^8$	0.1237	Boutrou et al., 2013
QEPVLGPVRGPFPIIV	β-casein f(194-209)	ACEi	$7.53x10^9 \pm 1.96x10^8$	$1.16x10^{10} \pm 5.14x10^8$	0.0044	Lu et al., 2016
EPVLGPVRGPFP	β-casein f(195-206)	ACEi	$3.79x10^8 \pm 3.38x10^7$	$4.90x10^8 \pm 4.07x10^7$	0.0219	Hayes et al., 2007
RPKHPIKHQ	αS1-casein f(1-9)	ACEi	$7.10x10^9 \pm 6.63x10^8$	$1.73x10^{10} \pm 2.66x10^9$	0.0032	Saito et al., 2000
ENLLRF	αS1-casein f(18-24)	ACEi	$8.23x10^8 \pm 1.76x10^8$	$1.46x10^9 \pm 2.66x10^8$	0.0226	Boutrou et al., 2013

FVAPFPEVF	αS1-casein f(24-32)	ACEi	$7.27x10^8 \pm 1.16x10^8$	$1.80x10^9 \pm 6.93x10^7$	0.0039	Boutrou et al., 2013
VAPFPEVF	αS1-casein f(25-32)	ACEi	$3.17x10^8 \pm 3.39x10^7$	$1.48x10^9 \pm 1.37x10^8$	0.0003	Boutrou et al., 2013
TKVIPYVRYL	αS2-casein f(198-207)	Antimicrobial	$1.62x10^8 \pm 3.75x10^7$	$5.30x10^7 \pm 2.11x10^7$	0.0180	Alvarez-Ordóñez et al., 2013

Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.
[a]Amounts were calculated by measuring the area under the peak (AUP) from the extracted ion chromatograms (EIC) obtained for each peptide and AUP values were normalized to the total peptide content of the milk hydrolysates. Values are means ± standard deviation. Statistically significant differences between PRA205 and PRA331 samples were calculated by Student's t-test ($P<0.05$).


PRA331
PRA205 Bioactive
29
(14%)
0
(0%)
103
(49.8%)
0
(0%)
0
(0%)
51
(24.6%)
0
(0%)
0
(0%)
0
(0%)
21
(10.1%)
3
(1.4%)
0
(0%)
0
(0%)
0
(0%)
0
(0%)
PRA205
PRA331 Bioactive

	RPKHPIKHQGLPQEVLNENLLRF
PRA205	↑ ↑ ↑ ↑ ↑ ↑ ↑
PRA331	↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEE

PRA205 ↑↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑

PRA331 ↑↑ ↑ ↑ ↑ ↑

QQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQ

PRA205 ↑ ↑ ↑ ↑↑↑↑ ↑ ↑

PRA331 ↑ ↑ ↑ ↑↑↑↑ ↑ ↑ ↑

NIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKH

PRA205 ↑↑ ↑ ↑ ↑↑ ↑ ↑ ↑ ↑ ↑ ↑↑ ↑ ↑↑

PRA331 ↑↑ ↑ ↑ ↑ ↑↑ ↑ ↑ ↑ ↑ ↑ ↑↑ ↑↑

KEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQ

PRA205 ↑↑ ↑↑ ↑ ↑↑↑↑ ↑↑↑↑

PRA331 ↑↑ ↑↑ ↑ ↑↑↑↑ ↑↑↑↑

SWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVP

PRA205 ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑↑↑↑

PRA331 ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑↑↑↑

QKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

PRA205 ↑ ↑ ↑ ↑↑↑↑↑↑ ↑ ↑↑↑ ↑ ↑ ↑

PRA331 ↑ ↑ ↑ ↑ ↑↑↑↑↑ ↑ ↑↑↑ ↑ ↑ ↑

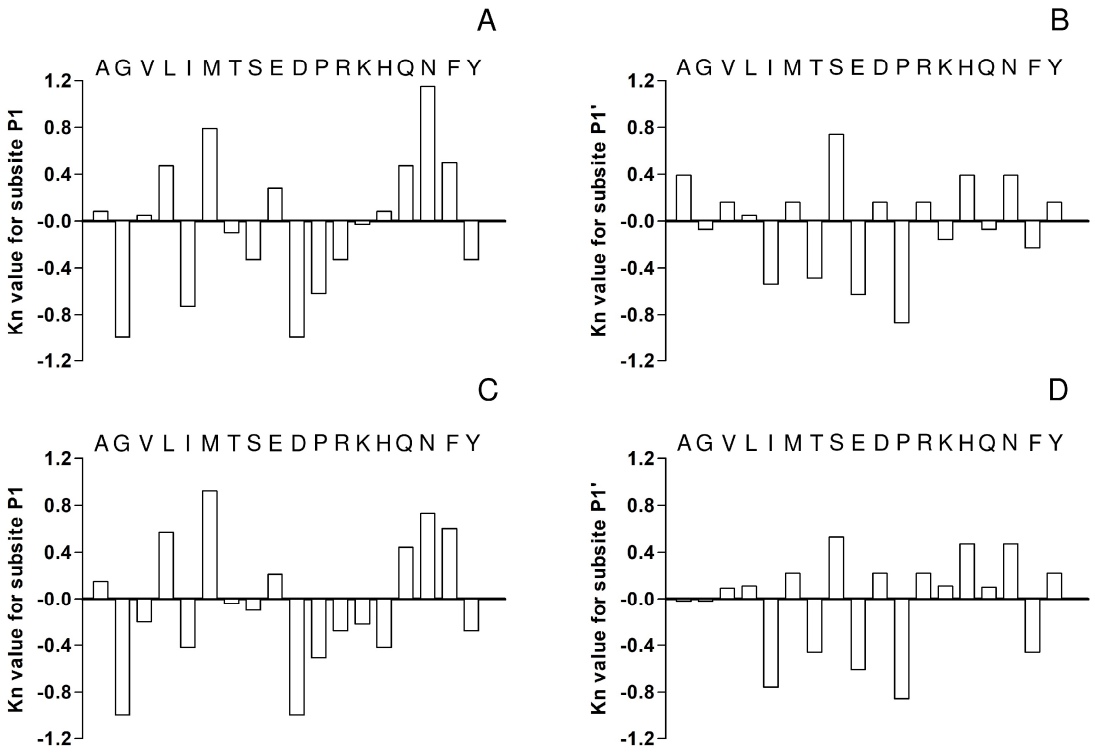

A
B
C
D
A G V L I M T S E D P R K H Q N F Y
Kn value for subsite P1
Kn value for subsite P1'
1.2
0.8
0.4
-0.0
-0.4
-0.8
-1.2

Table 1. Cleavage occurrence and cleavage probability (*%P*) produced by *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 cell-envelope proteinase on β-casein at different amino acids in the P1 and P1'subsites.

	Lb. casei PRA205			*Lb. rhamnosus* PRA331		
Amino acids[a]	**Number of residues**	**P1 subsite** Number of cleaved bond[b] (%P1[c])	**P1' subsite** Number of cleaved bond[b] (%P1'[c])	**Number of residues**	**P1 subsite** Number of cleaved bond[b] (%P1[c])	**P1' subsite** Number of cleaved bond[b] (%P1'[c])
Aliphatic amino acids						
A	5	2 (40.0)	3 (60.0)	5	2 (40.0)	2 (40.0)
G	5	0 (0)	2 (40.0)	5	0 (0)	2 (40.0)
V	18	7 (38.9)	9 (50.0)	18	5 (27.8)	8 (44.4)
L	22	12 (54.6)	10 (45.5)	22	12 (54.6)	10 (45.5)
I	10	1 (10.0)	2 (20.0)	10	2 (20.0)	1 (10.0)
M	6	4 (66.7)	3 (50.0)	6	4 (66.7)	3 (50.0)
Polar un-charged amino acids						
T	9	3 (33.3)	2 (22.2)	9	3 (33.3)	2 (22.2)
S	16	4 (25.0)	12 (75.0)	16	5 (31.3)	10 (61.5)
E	19	9 (47.4)	3 (15.8)	19	8 (42.1)	3 (15.8)
D	4	0 (0)	2 (50.0)	4	0 (0)	2 (50.0)
P	35	5 (14.3)	2 (5.7)	35	6 (17.1)	2 (5.7)
Positively charged amino acids						
R	4	1 (25.0)	2 (50.0)	4	1 (25.0)	2 (50.0)
K	11	4 (36.4)	4 (36.4)	11	3 (27.3)	5 (45.5)
H	5	2 (40.0)	3 (60.0)	5	1 (20.0)	3 (60.0)
Negatively charged amino acids						
Q	20	11 (55.0)	8 (40.0)	20	10 (50.0)	9 (45.0)
N	5	4 (80.0)	3 (60.0)	5	3 (60.0)	3 (60.0)
Aromatic amino acids						
F	9	5 (55.6)	3 (33.3)	9	5 (55.7)	2 (22.2)
Y	4	1 (25.0)	2 (50.0)	4	1 (25.0)	2 (50.0)

[a]One code letter was used for amino acid nomenclature. The amino acid C is not present in the sequence of β-casein, while the amino acid W was omitted from the analysis since it occurs once in the β-casein sequence.
[b]The cleaved bonds are reported in **Figure 3**.
[c]See materials and methods section for the calculation of the *%P1* and *%P1'* cleavage probability.

Table 2. Peptides with previously demonstrated bioactivity identified in the milk hydrolysates by whole cells of *Lactobacillus casei* PRA205 or *Lactobacillus rhamnosus* PRA331.

Sequence	*Fragment*	*Bioactivity*	*PRA205 relative amount[a] (±SD)*	*PRA331 relative amount[a] (±SD)*	*P-value*	*Reference*
LNVPGEIVE	β-casein f(6-14)	ACEi	$1.13x10^9 \pm 1.26x10^8$	$1.30x10^9 \pm 4.21x10^7$	0.0876	Gobbetti et al., 2000
VPGEIVE	β-casein f(8-14)	DPPIV-inhibitor	$4.30x10^8 \pm 3.38x10^7$	n.d.	/	Nongonierma et al., 2016
DKIHPF	β-casein f(47-52)	ACEi	$2.37x10^{10} \pm 3.37x10^9$	$2.36x10^{10} \pm 6.25x10^8$	0.4925	Gobbetti et al., 2000
LVYPFPGPIPNSLPQ	β-casein f(58-72)	ACE-inhibitor	$3.44x10^8 \pm 3.89x10^7$	n.d.	/	Smacchi et al., 2008
VYPFPGPIPN	β-casein f(59-68)	ACEi Antioxidant Opioid agonist	$8.19x10^9 \pm 7.04x10^8$	$1.62x10^{10} \pm 4.17x10^8$	0.0004	Eisele et al., 2013
YPFPGPIPN	β-casein f(60-68)	ACEi DPPIV-inhibitor Opioid agonist	$3.34x10^9 \pm 1.30x10^8$	$1.03x10^{10} \pm 1.89x10^9$	0.0030	Saito et al., 2000
NIPPLTQTPV	β-casein f(73-82)	ACEi	$9.51x10^9 \pm 5.72x10^8$	$5.96x10^9 \pm 4.64x10^8$	0.0027	Gobbetti et al., 2000
NLHLPLP	β-casein f(132-138)	ACEi	$1.94x10^9 \pm 2.09x10^8$	$7.81x10^8 \pm 1.22x10^8$	0.0031	Kohmura et al., 1989
NLHLPLPLL	β-casein f(132-140)	ACEi	$8.78x10^8 \pm 3.33x10^7$	$5.92x10^8 \pm 1.39x10^7$	0.0037	Robert et al., 2004
LHLPLP	β-casein f(133-138)	ACEi	$3.40x10^8 \pm 3.47x10^7$	$7.64x10^8 \pm 6.41x10^7$	0.0072	Kohmura et al., 1989
LHLPLPL	β-casein f(133-139)	ACEi	$2.55x10^9 \pm 2.85x10^8$	$3.06x10^9 \pm 2.19x10^8$	0.0920	Quiros et al., 2007
SQSKVLPVPQ	β-casein f(166-175)	ACEi	$3.49x10^8 \pm 3.51x10^7$	$6.14x10^8 \pm 7.03x10^7$	0.0050	Hayes et al., 2007
SKVLPVPQ	β-casein f(168-175)	ACEi	$8.32x10^8 \pm 1.07x10^8$	$1.07x10^9 \pm 7.70x10^7$	0.0380	Yamamoto et al., 1994
KVLPVPQ	β-casein f(169-175)	ACEi	$6.05x10^9 \pm 5.74x10^8$	$1.40x10^{10} \pm 1.76x10^9$	0.0022	Maeno et al., 1996
RDMPIQAF	β-casein f(183-190)	ACEi	$7.64x10^9 \pm 2.57x10^8$	$1.33x10^9 \pm 8.06x10^7$	<0.0001	Yamamoto et al., 1994
LYQEPVLGPVRGPFPIIV	β-casein f(192-209)	Immunomodulator	$3.19x10^8 \pm 4.13x10^7$	n.d.		Boutrou et al., 2013
YQEPVLGPVRGPFPIIV	β-casein f(193-209)	Immunomodulator	$1.69x10^9 \pm 3.86x10^8$	$1.21x10^9 \pm 1.57x10^8$	0.1237	Boutrou et al., 2013
QEPVLGPVRGPFPIIV	β-casein f(194-209)	ACEi	$7.53x10^9 \pm 1.96x10^8$	$1.16x10^{10} \pm 5.14x10^8$	0.0044	Lu et al., 2016
EPVLGPVRGPFP	β-casein f(195-206)	ACEi	$3.79x10^8 \pm 3.38x10^7$	$4.90x10^8 \pm 4.07x10^7$	0.0219	Hayes et al., 2007
RPKHPIKHQ	αS1-casein f(1-9)	ACEi	$7.10x10^9 \pm 6.63x10^8$	$1.73x10^{10} \pm 2.66x10^9$	0.0032	Saito et al., 2000
ENLLRF	αS1-casein f(18-24)	ACEi	$8.23x10^8 \pm 1.76x10^8$	$1.46x10^9 \pm 2.66x10^8$	0.0226	Boutrou et al., 2013

FVAPFPEVF	αS1-casein f(24-32)	ACEi	$7.27x10^8 \pm 1.16x10^8$	$1.80x10^9 \pm 6.93x10^7$	0.0039	Boutrou et al., 2013
VAPFPEVF	αS1-casein f(25-32)	ACEi	$3.17x10^8 \pm 3.39x10^7$	$1.48x10^9 \pm 1.37x10^8$	0.0003	Boutrou et al., 2013
TKVIPYVRYL	αS2-casein f(198-207)	Antimicrobial	$1.62x10^8 \pm 3.75x10^7$	$5.30x10^7 \pm 2.11x10^7$	0.0180	Alvarez-Ordóñez et al., 2013

Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV. Only peptides found from the literature to have 100% homology to known functional peptides were reported in the **Table**. The complete list of identified peptides can be found in Supplementary on line **Tables S1-S8**.

[a]Amounts were calculated by measuring the area under the peak (AUP) from the extracted ion chromatograms (EIC) obtained for each peptide and AUP values were normalized to the total peptide content of the milk hydrolysates as described in section 2.6. Values are means ± standard deviation. Statistically significant differences between PRA205 and PRA331 samples were calculated by Student's t-test ($P<0.05$).

Figure S1. β-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Figure S2. αS1-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Figure S3. αS2-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to

peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Figure S4. κ-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Lactobacillus casei PRA205

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPP

LTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQ

PHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

Lactobacillus rhamnosus PRA331

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPP

LTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQ

PHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

Lactobacillus casei PRA205

RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSV

EQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFR

QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW

Lactobacillus rhamnosus PRA331

RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSV

EQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFR

QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW

Lactobacillus casei PRA205

KNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDK

HYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVFTKK

TKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL

Lactobacillus rhamnosus PRA331

KNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDK

HYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVFTKK

TKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL

Lactobacillus casei PRA205

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQ

WQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDS

PEVIESPPEINTVQVTSTAV

Lactobacillus rhamnosus PRA331

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQ

WQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDS

PEVIESPPEINTVQVTSTAV

Table S1. β-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells[a].

Sequence[b]	*Observed mass (m/z)*[c]	*Calculated mass*[d]	*Fragment*	*Bioactivity*[e]	*Reference*
LNVPGEIVE	969.5562	968.5179	f(6-14)	ACEi	Gobbetti et al., 2000
NVPGEIVE	856.4430	855.4338	f(7-14)	/	/
VPGEIVE	742.3943	741.3909	f(8-14)	DPPIV-inhibitor	Nongonierma et al., 2016
SITRIN	352.1970	702.4024	f(22-27)	/	/
KKIEKF	396.7429	791.4905	f(28-33)	/	/
KKIEKFQ	460.7709	919.5491	f(28-34)	/	/
KKIEKFQS(phospho)E	608.8120	1215.5900	f(28-36)	/	/
KKIEKFQS(phospho)EE	673.3181	1344.6326	f(28-37)	/	/
IEKFQS(phospho)EE	545.2067	1088.4427	f(30-37)	/	/
DKIHPF	756.3786	755.3966	f(47-52)	ACEi	Gobbetti et al., 2000
DKIHPFAQTQ	592.7785	1183.5986	f(47-56)	/	/
SLVYPFPGPIPN	650.8484	1299.6863	f(57-68)	/	/
SLVYPFPGPIPNSLPQ	863.4501	1724.9138	f(57-72)	/	/
LVYPFPGPIPN	1213.6762	1212.6543	f(58-68)	/	/
LVYPFPGPIPNSLPQ	819.9604	1724.9138	f(58-72)	ACEi	Smacchi et al., 2008
VYPFPGPIPN	1100.5568	1099.5702	f(59-68)	ACEi Antioxidant Opioid agonist	Eisele et al., 2013
VYPFPGPIPNSLPQ	763.4073	1524.7977	f(59-72)	/	/
YPFPGPIPN	1001.5291	1000.5018	f(60-68)	ACEi DPPIV-inhibitor Opioid agonist	Saito et al., 2000
SLPQNIPPL	978.5771	977.5546	f(69-77)	/	/
SLPQNIPPLTQTPVVVPPFLQPEVM(ox)	919.8153	2755.4823	f(69-93)	/	/
NIPPLTQTPV	1079.6176	1078.6023	f(73-82)	ACEi	Gobbetti et al., 2000

NIPPLTQTPVVVPPF	809.9563	1617.9131	f(73-87)	/	/
NIPPLTQTPVVVPPFLQPEVM	772.7640	2315.2599	f(73-93)	/	/
TQTPV	545.2884	544.2857	f(78-82)	/	/
TQTPVVVPPF	1084.5754	1083.5965	f(78-87)	/	/
TQTPVVVPPFLQPE	776.4502	1550.8345	f(78-91)	/	/
TQTPVVVPPFLQPEVM	891.4896	1780.9434	f(78-93)	/	/
TQTPVVVPPFLQPEVMGV	969.5306	1937.0333	f(78-95)	/	/
TQTPVVVPPFLQPEVMGVSKVKEAMAP	960.5079	2878.5337	f(78-104)	/	/
QTPVVVPPFLQPEVM	840.9568	1679.8957	f(79-93)	/	/
PVVVPPFLQPEVM	726.4071	1450.7894	f(81-93)	/	/
VVPPFLQPE	1025.6040	1024.5593	f(83-91)	/	/
VVPPFLQPEVM	1255.6987	1254.6682	f(83-93)	/	/
VPPFLQPE	463.7369	925.4909	f(84-91)	/	/
VPPFLQPEVM	578.7970	1155.5968	f(84-93)	/	/
PPFLQPE	827.4361	826.4225	f(85-91)	/	/
LQPEVM	716.3580	715.3575	f(88-93)	/	/
AMAPKHKEMPFPKYPVEPF	748.7340	2243.1271	f(101-119)	/	/
MAPKHKEMPFPKYPVEPF	725.0321	2172.0900	f(102-119)	/	/
APKHKEMPFPKYPVEPF	681.3579	2041.0495	f(103-119)	/	/
APKHKEMPFPKYPVEPFTESQ	622.5635	2486.2304	f(103-123)	/	/
KHKEMPFPKYPVEPF	625.3218	1872.9596	f(105-119)	/	/
HKEMPFPKYPVEPF	582.6372	1744.8647	f(106-119)	/	/
HKEMPFPKYPVEPFTESQ	730.9973	2190.0456	f(106-123)	/	/
EMPFPKYPVEP	667.3054	1332.6424	f(108-118)	/	/
EMPFPKYPVEPF	740.8620	1479.7108	f(108-119)	/	/
MPFPKYPVEP	602.8135	1203.5998	f(109-118)	/	/

MPFPKYPVEPF	676.3415	1350.6682	f(109-119)	/	/
NLHLPLP	402.2601	801.4701	f(132-138)	ACEi	Kohmura et al., 1989
NLHLPLPL	916.5616	915.5542	f(132-139)	/	/
NLHLPLPLL	515.3168	1028.6382	f(132-140)	ACEi	Robert et al., 2004
NLHLPLPLLQ	579.3496	1156.6968	f(132-141)	/	/
NLHLPLPLLQS	622.8851	1243.7288	f(132-142)	/	/
NLHLPLPLLQSW	715.9295	1429.8082	f(132-143)	/	/
LHLPLP	345.2209	688.4272	f(133-138)	ACEi	Kohmura et al., 1989
LHLPLPL	401.7546	801.5112	f(133-139)	ACEi	Quiros et al., 2007
LHLPLPLLQ	1043.6493	1042.6539	f(133-141)	/	/
LHLPLPLLQS	565.8369	1129.6859	f(133-142)	/	/
LHLPLPLLQSW	658.9507	1315.7652	f(133-143)	/	/
HLPLPLLQ	465.7873	929.5698	f(134-141)	/	/
HLPLPLLQSW	602.3328	1202.6812	f(134-143)	/	/
LPLPLLQ	793.5148	792.5109	f(135-141)	/	/
LPLPLLQSW	1066.6182	1065.6223	f(135-143)	/	/
WMHQPHQPLPPT	490.2347	1467.7081	f(143-154)	/	/
WMHQPHQPLPPTVM	566.9374	1697.8170	f(143-156)	/	/
MHQPHQPLPPT	641.8116	1281.6288	f(144-154)	/	/
MHQPHQPLPPTVM	756.8776	1511.7377	f(144-156)	/	/
MHQPHQPLPPTVMFPPQ	661.3254	1982.3793	f(144-160)	/	/
HQPHQPLPPT	576.2953	1150.5883	f(145-154)	/	/
HQPHQPLPPTVM	691.3551	1370.6972	f(145-156)	/	/
HQPHQPLPPTVMFPPQ	617.6508	1849.9298	f(145-160)	/	/
QPHQPLPPTVM	622.8084	1243.6383	f(146-156)	/	/
VMFPPQ	359.6717	717.3520	f(155-160)	/	/

VMFPPQSVL	1017.5629	1016.5365	f(155-163)	/	/
FPPQSVL	787.4301	786.4276	f(157-163)	/	/
SQSKVLPVPQ	541.8009	1071.6132	f(166-175)	ACEi	Hayes et al., 2007
QSKVLPVPQ	995.5916	994.5811	f(167-175)	/	/
QSKVLPVPQKAVPYPQR	484.5272	1934.1102	f(167-182)	/	/
SKVLPVPQ	434.2542	866.5226	f(168-175)	ACEi	Yamamoto et al., 1994
KVLPVPQ	390.7414	779.4905	f(169-175)	ACEi	Maeno et al., 1996
VLPVPQ	652.3973	651.3956	f(170-175)	/	/
KAVPYPQ	401.7171	801.4385	f(176-182)	/	/
KAVPYPQR	479.7656	957.5396	f(176-183)	/	/
RDMPIQA	415.7139	829.4116	f(183-189)	/	/
RDMPIQAF	489.2411	976.4800	f(183-190)	ACEi	Yamamoto et al., 1994
RDMPIQAFLL	602.3328	1202.6481	f(183-192)	/	/
LYQEPVL	861.4568	860.4644	f(192-198)	/	/
LYQEPVLGPVRGPFP	834.9509	1667.9035	f(192-206)	/	/
LYQEPVLGPVRGPFPIIV	997.5960	1993.1401	f(192-209)	Immunomodulator	Boutrou et al., 2013
YQEPVL	748.3554	747.3803	f(193-198)	/	/
YQEPVLGPVRGPFP	778.4137	1554.8195	f(193-206)	/	/
YQEPVLGPVRGPFPIIV	941.0462	1880.0560	f(193-209)	Immunomodulator	Boutrou et al., 2013
QEPVL	585.3251	584.3170	f(194-198)	/	/
QEPVLGPVRGPFP	696.8883	1391.7561	f(194-206)	/	/
QEPVLGPVRGPFPII	809.9563	1617.9243	f(194-208)	/	/
QEPVLGPVRGPFPIIV	859.4987	1716.9927	f(194-209)	ACEi	Lu et al., 2016
EPVLGPVRGPFP	632.8584	1263.6976	f(195-206)	ACEi	Hayes et al., 2007
EPVLGPVRGPFPIIV	795.4756	1588.9341	f(195-209)	/	/
VLGPVRGPFPIIV	682.4140	1362.8388	f(197-209)	/	/

LGPVRGPFPIIV	632.8902	1263.7703	f(198-209)	/	/
GPVRGPFP	413.7223	825.4497	f(199-206)	/	/
GPVRGPFPII	526.8104	1051.6179	f(199-208)	/	/
GPVRGPFPIIV	576.3457	1150.6863	f(199-209)	/	/
RGPFPIIV	449.7709	897.5436	f(202-209)	/	/

[a]Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S2. αS1-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells[a].

Sequence[b]	*Observed mass (m/z)*[c]	*Calculated mass*[d]	*Fragment*	*Bioactivity*[e]	*Reference*
RPKHPIKH	338.2150	1011.6090	f(1-8)	/	/
RPKHPIKHQ	570.8322	1139.6676	f(1-9)	ACEi	Saito et al., 2000
RPKHPIKHQGLPQ	512.6430	1534.8844	f(1-13)	/	/
RPKHPIKHQGLPQEVLN	498.5285	1990.1224	f(1-17)	/	/
KHPIKHQ	296.4995	886.5137	f(3-9)	/	/
GLPQEVLNE	499.7432	997.5080	f(10-18)	/	/
ENLLRF	396.2126	790.4337	f(18-24)	ACEi	Boutrou et al., 2013
FVAPFPE	806.3771	805.4010	f(24-30)	/	/
FVAPFPEVF	1052.5566	1051.5379	f(24-32)	ACEi	Boutrou et al., 2013
FVAPFPEVFGKE	683.8712	1365.6969	f(24-35)	/	/
VAPFPE	659.3117	658.3326	f(25-30)	/	/
VAPFPEVF	453.2311	904.4695	f(25-32)	ACEi	Boutrou et al., 2013
VAPFPEVFGK	545.7962	1089.5859	f(25-34)	/	/
VAPFPEVFGKE	610.2613	1218.6285	f(25-35)	/	/
VFGKEKV	403.7262	805.4698	f(31-37)	/	/
VFGKEKVN	307.4989	919.5127	f(31-38)	/	/
VFGKEKVNEL	581.8127	1161.6394	f(31-40)	/	/
S(phospho)VEQKHIQ	524.7428	1047.4750	f(75-82)	/	/
RLKKYKVPQ	387.2312	1158.7237	f(100-108)	/	/
KKYKVPQ	445.7761	889.5385	f(102-108)	/	/
KYKVPQ	381.7173	761.4436	f(103-108)	/	/
LEIVPN	684.3778	683.3854	f(109-114)	/	/
S(phospho)AEELRH	461.1781	920.3753	f(115-121)	/	/
SMKEGIH	401.1859	800.3851	f(122-128)	/	/

KEGIHAQ	391.7040	781.4082	f(124-130)	/	/
AQQKEPM	416.1894	830.3956	f(139-135)	/	/
QKEPMIGVN	508.2554	1014.5168	f(131-139)	/	/
FSDIPNPIGSE	1175.5582	1174.5506	f(179-189)	/	/
FSDIPNPIGSEN	645.2999	1288.5935	f(179-190)	/	/
FSDIPNPIGSENSE	753.3396	1504.6682	f(179-192)	/	/
FSDIPNPIGSENSEK	817.3947	1632.7631	f(179-193)	/	/
SDIPNPIGSENSE	679.7936	1357.5997	f(180-192)	/	/
DIPNPIGSENSE	636.2773	1270.5677	f(181-192)	/	/

[a]Abbreviation is: ACEi, angiotensin converting enzyme-inhibitory.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S3. αS2-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells.

Sequence[a]	*Observed mass (m/z)*[b]	*Calculated mass*[c]	*Fragment*	*Bioactivity*[d]	*Reference*
SIIS(phospho)QETYK	574.7641	1147.5162	f(13-21)	/	/
RNAVPITPT	484.7610	967.5451	f(114-122)	/	/
NAVPITPT	812.4495	811.4440	f(115-122)	/	/
NAVPITPTLNRE	662.8478	1323.7146	f(115-126)	/	/
AVPITPT	698.4023	697.4010	f(116-122)	/	/
AVPITPTLNRE	605.8537	1209.6717	f(116-126)	/	/
LNREQLS(phospho)TS(phospho)EE	733.2800	1464.5534	f(123-133)	/	/
NSKKTVD	396.2126	790.4185	f(134-140)	/	/
MES(phospho)TEVFTK	576.2371	1150.4617	f(141-149)	/	/
TKKTKLTE	474.7895	947.5651	f(148-155)	/	/
TKVIPYVRYL	417.9110	1250.7387	f(198-207)	Antimicrobial	Alvarez-Ordóñez et al., 2013

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$.
[c]The calculated mass is in Da.
[d]Potential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S4. κ-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells.

Sequence[a]	*Observed mass (m/z)*[b]	*Calculated mass*[c]	*Fragment*	*Bioactivity*[d]	*Reference*
FSDKIA	340.6720	679.3541	f(18-23)	/	/
KYIPIQY	462.7570	923.5116	f(24-30)	/	/
KYIPIQYVL	568.8331	1135.6641	f(24-32)	/	/
SRYPSYGLN	528.7613	1055.5036	f(33-41)	/	/
YYQQKPV	463.2334	924.4705	f(42-48)	/	/
YYQQKPVAL	555.2851	1108.5917	f(42-50)	/	/
YYQQKPVALIN	668.8545	1335.7187	f(42-52)	/	/
YYQQKPVALINN	725.8879	1449.7616	f(42-53)	/	/
QKPVALINN	498.7884	995.5764	f(45-53)	/	/
NQFLPYPYYAKPA	786.4022	1570.7820	f(53-65)	/	/
QFLPYPYYAKPA	729.3616	1456.7391	f(54-65)	/	/
FLPYPYYAKPA	665.3372	1328.7805	f(55-65)	/	/
LPYPYYAKPA	591.8096	1181.6121	f(56-65)	/	/
YAKPA	275.1487	548.2958	f(61-65)	/	/
AVRSPA	300.6684	599.3391	f(66-71)	/	/
AVRSPAQIL	477.7794	953.5658	f(66-74)	/	/
AVRSPAQILQ	541.8009	1081.6244	f(66-75)	/	/
ARHPHPHLS	351.1777	1050.5471	f(96-104)	/	/
ARHPHPHLSF	400.2009	1197.6156	f(96-105)	/	/
ARHPHPHLSFM	443.8951	1328.6560	f(96-106)	/	/
DKTEIPTIN	515.7586	1029.5342	f(116-123)	/	/
KTEIPTIN	458.2511	914.5073	f(117-123)	/	/
EIPTIN	686.3773	685.3646	f(118-123)	/	/
TIASGEPT	775.3848	774.3759	f(124-131)	/	/

VATLEDS(phospho)PE	520.7034	1039.4111	f(143-152)	/	/
VIESPPEIN	997.5065	996.5128	f(152-160)	/	/
SPPEIN	656.3215	655.3177	f(155-160)	/	/
SPPEINTVQ	984.5185	983.4924	f(155-163)	/	/
VTSTAV	577.3151	576.3119	f(164-169)	/	/

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$.
[c]The calculated mass is in Da.
[d]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

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Table S5. β-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells[a].

Sequence[b]	*Observed mass (m/z)*[c]	*Calculated mass*[d]	*Fragment*	*Bioactivity*[e]	*Reference*
LNVPGEIVE	485.2516	968.5179	f(6-14)	ACEi	Gobbetti et al., 2000
NVPGEIVE	856.4256	855.4338	f(7-14)	/	/
SITRIN	352.1995	702.4024	f(22-27)	/	/
KKIEKF	396.7440	791.4905	f(28-33)	/	/
KKIEKFQS(phospho)E	608.8120	1215.5900	f(28-36)	/	/
DKIHPF	378.6905	755.3966	f(47-52)	ACEi	Gobbetti et al., 2000
DKIHPFA	414.2081	826.4337	f(47-53)	/	/
SLVYPFPGPIPN	1300.6991	1299.6863	f(57-68)	/	/
LVYPFPGPIPN	1213.6643	1212.6543	f(58-68)	/	/
VYPFPGPIPN	1100.5503	1099.5702	f(59-68)	ACEi Antioxidant, Opioid	Eisele et al., 2013
VYPFPGPIPNSLPQ	763.3809	1524.7977	f(59-72)	/	/
YPFPGPIPN	1001.5294	1000.5018	f(60-68)	ACEi DPPIV-inhibitor, Opioid	Saito et al., 2000
SLPQNIPPL	978.5704	977.5546	f(69-77)	/	/
SLPQNIPPLTQTPV	752.9436	1503.8297	f(69-82)	/	/
SLPQNIPPLTQTPVVVPPFLQPEVM	1371.2513	2740.4874	f(69-93)	/	/
QNIPPLTQTPV	604.3167	1206.6608	f(72-82)	/	/
QNIPPLTQTPVVVPPF	874.0031	1745.9716	f(72-87)	/	/
QNIPPLTQTPVVVPPFLQPE	738.7323	2213.2096	f(72-91)	/	/
QNIPPLTQTPVVVPPFLQPEVM	815.4550	2443.3185	f(72-93)	/	/
QNIPPLTQTPVVVPPFLQPEVMGVS	896.4847	2686.4404	f(69-96)	/	/
NIPPLTQTPV	540.3031	1078.6023	f(73-82)	ACEi	Gobbetti et al., 2000

NIPPLTQTPVVVPPF	809.9655	1617.9131	f(73-87)	/	/
NIPPLTQTPVVVPPFLQPEVM	1158.6488	2315.2599	f(73-93)	/	/
TQTPVVVPPF	542.7930	1083.5965	f(78-87)	/	/
TQTPVVVPPFL	599.3400	1196.6805	f(78-88)	/	/
TQTPVVVPPFLQPE	776.4225	1550.8345	f(78-91)	/	/
TQTPVVVPPFLQPEVM	891.4927	1780.9434	f(78-93)	/	/
TQTPVVVPPFLQPEVMGVS	1013.0422	2024.0653	f(78-96)	/	/
TQTPVVVPPFLQPEVMGVSKVKEAMAP	960.5032	2878.5337	f(78-104)	/	/
QTPVVVPPFLQPE	725.8957	1449.7868	f(79-91)	/	/
QTPVVVPPFLQPEVM	840.9478	1679.8957	f(79-93)	/	/
PVVVPPFLQPE	611.3391	1220.6805	f(81-91)	/	/
PVVVPPFLQPEVM	726.3960	1450.7894	f(81-93)	/	/
VVPPFLQPE	1025.5809	1024.5593	f(83-91)	/	/
VVPPFLQPEVM	1255.6756	1254.6682	f(83-93)	/	/
VPPFLQPEVM	578.7956	1155.5998	f(84-93)	/	/
PEVMGVSKVKEAMAPK	567.6384	1700.9074	f(90-105)	/	/
VMGSKVKEA	349.8603	1046.5794	f(92-101)	/	/
MAPKHKEMPFPKYPVEPF	725.0518	2172.0900	f(102-119)	/	/
APKHKEMPFPKYPVEPF	681.3265	2041.0495	f(103-119)	/	/
HKEMPFPKYPVEPF	582.6204	1744.8647	f(106-119)	/	/
EMPFPKYPVEPF	740.8436	1479.7108	f(108-119)	/	/
MPFPKYPVEP	602.8002	1203.5998	f(109-118)	/	/
MPFPKYPVEPF	676.3260	1350.6682	f(109-119)	/	/
MPFPKYPVEPFTE	791.3602	1580.7585	f(109-121)	/	/
NLHLPLP	402.2289	802.4701	f(132-138)	ACEi	Kohmura et al., 1989
NLHLPLPL	458.7739	915.5542	f(132-139)	/	/

NLHLPLPLL	515.3360	1028.6632	f(132-140)	ACEi	Robert et al., 2004
NLHLPLPLLQ	579.3461	1156.6968	f(132-141)	/	/
NLHLPLPLLQS	622.8557	1243.7288	f(132-142)	/	/
NLHLPLPLLQSW	715.8693	1429.8082	f(132-143)	/	/
LHLPLP	345.2061	688.4272	f(133-138)	ACEi	Kohmura et al., 1989
LHLPLPL	401.7568	801.5112	f(133-139)	ACEi	Quiros et al., 2007
LHLPLPLLQ	522.3205	1042.6539	f(133-141)	/	/
LHLPLPLLQS	565.8474	1129.6859	f(133-142)	/	/
LHLPLPLLQSW	658.8784	1315.7652	f(133-143)	/	/
HLPLPL	345.2061	688.4272	f(134-139)	/	/
HLPLPLLQSW	602.3478	1202.6812	f(134-143)	/	/
LPLPLLQ	793.5105	792.5109	f(135-141)	/	/
LPLPLLQSW	533.8107	1065.6223	f(135-143)	/	/
WMHQPHQPLPPTVMFPPQ	723.3596	2167.0496	f(143-160)	/	/
MHQPHQPLPPT	641.8081	1281.6288	f(144-154)	/	/
MHQPHQPLPPTVM	504.9035	1511.7377	f(144-156)	/	/
MHQPHQPLPPTVMFPPQ	661.3167	1980.9703	f(144-160)	/	/
HQPHQPLPPT	576.2926	1150.5883	f(145-154)	/	/
HQPHQPLPPTVM	461.2329	1380.6972	f(145-156)	/	/
HQPHQPLPPTVMFPPQ	617.6437	1849.9298	f(145-160)	/	/
FPPQSVL	787.4370	786.4272	f(157-163)	/	/
SQSKVLPVPQ	541.8019	1081.6132	f(166-175)	ACEi	Hayes et al., 2007
QSKVLPVPQ	498.2873	994.5811	f(167-175)	/	/
SKVLPVPQ	434.2558	866.5226	f(168-175)	ACEi	Yamamoto et al., 1994
KVLPVPQ	780.4975	779.4905	f(169-175)	ACEi	Maeno et al., 1996
VLPVPQ	652.3929	651.3956	f(170-175)	/	/

KAVPYPQ	401.7177	801.4385	f(176-182)	/	/
KAVPYPQRDMPI	707.8531	1413.7438	f(176-186)	/	/
RDMPIQAF	489.2344	976.4800	f(183-190)	ACEi	Yamamoto et al., 1994
RDMPIQAFL	545.7702	1089.5641	f(183-191)	/	/
RDMPIQAFLL	602.3312	1202.6481	f(183-192)	/	/
LYQEPVL	861.4602	860.4644	f(192-198)	/	/
YQEPVL	748.3849	747.3803	f(193-198)	/	/
YQEPVLGPVRGPFP	778.4100	1554.8195	f(193-206)	/	/
YQEPVLGPVRGPFPIIV	941.0424	1880.0560	f(193-209)	Immunomodulator	Boutrou et al., 2013
QEPVLGPVRGPFP	686.8812	1391.7561	f(194-206)	/	/
QEPVLGPVRGPFPIIV	859.5135	1716.9927	f(194-209)	ACEi	Lu et al., 2016
EPVLGPVRGPFP	632.8351	1263.6976	f(195-206)	ACEi	Hayes et al., 2007
EPVLGPVRGPFPIIV	795.4796	1588.9341	f(195-209)	/	/
VLGPVRGPFPIIV	682.4057	1362.8388	f(197-209)	/	/
LGPVRGPFPIIV	632.8861	1263.7703	f(198-209)	/	/
GPVRGPFP	413.7160	825.4497	f(199-206)	/	/
GPVRGPFPII	526.7979	1051.6179	f(199-208)	/	/
GPVRGPFPIIV	576.3487	1150.6863	f(199-209)	/	/
RGPFPIIV	449.7698	897.5436	f(202-209)	/	/

[a]Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S6. αS1-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells[a].

Sequence[b]	*Observed mass (m/z)*[c]	*Calculated mass*[d]	*Fragment*	*Bioactivity*[e]	*Reference*
RPKHPIKH	338.1924	1011.6090	f(1-8)	/	/
RPKHPIKHQ	380.8905	1139.6676	f(1-9)	ACEi	Saito et al., 2000
RPKHPIKHQGLPQ	512.6139	1534.8844	f(1-13)	/	/
RPKHPIKHQGLPQEVLN	498.5303	1990.1224	f(1-17)	/	/
KHPIKHQ	444.2507	886.5137	f(3-9)	/	/
GLPQEVL	755.4085	754.4298	f(10-16)	/	/
GLPQEVLNE	499.7582	997.5080	f(10-18)	/	/
ENLLRF	396.2095	790.4337	f(18-24)	ACEi	Boutrou et al., 2013
FVAPFPE	806.4074	805.4010	f(24-30)	/	/
FVAPFPEVF	1052.5164	1051.5379	f(24-32)	ACEi	Boutrou et al., 2013
FVAPFPEVFGKE	683.8608	1365.6969	f(24-35)	/	/
VAPFPE	659.3349	658.3326	f(25-30)	/	/
VAPFPEVF	905.4909	904.4695	f(25-32)	ACEi	Boutrou et al., 2013
VAPFPEVFGK	545.8130	1089.5859	f(25-34)	/	/
VAPFPEVFGKE	610.3173	1218.6285	f(25-35)	/	/
APFPEVF	806.4074	805.4010	f(26-32)	/	/
APFPEVFGKE	560.7993	1119.5601	f(26-35)	/	/
VFGKEKVN	460.7541	919.5127	f(31-38)	/	/
KKYKVPQ	445.7685	889.5385	f(102-108)	/	/
KYKVPQ	381.7245	761.4436	f(103-108)	/	/
LEIVPN	684.3640	683.3854	f(109-114)	/	/
S(phospho)AEELRH	461.1787	920.3753	f(115-121)	/	/
S(phospho)AEELRHSM	570.2206	1138.4478	f(115-123)	/	/
KEGIHAQ	391.6973	781.4082	f(124-130)	/	/

APSFSDIPNPIGSENSE	880.9123	1759.7901	f(176-192)	/	/
FSDIPNPIGSE	588.2765	1174.5506	f(179-189)	/	/
FSDIPNPIGSEN	645.3093	1288.5935	f(179-190)	/	/
FSDIPNPIGSENSE	753.3208	1504.6682	f(179-192)	/	/
IPNPIGSENSE	578.7531	1155.5408	f(182-192)	/	/

[a]Abbreviation is: ACEi, angiotensin converting enzyme-inhibitory.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S7. αS2-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331.

Sequence[a]	*Observed mass (m/z)*[b]	*Calculated mass*[c]	*Fragment*	*Bioactivity*[d]	*Reference*
SIIS(phospho)QETYK	574.7617	1147.5162	f(13-21)	/	/
NAVPITPT	812.4463	811.4440	f(115-122)	/	/
NAVPITPTLN	520.2697	1038.5710	f(115-124)	/	/
NAVPITPTLNRE	662.8581	1323.7146	f(115-126)	/	/
AVPITPT	698.4069	697.4010	f(116-122)	/	/
AVPITPTLNRE	605.8419	1209.6717	f(116-126)	/	/
MES(phospho)TEVFTK	576.2277	1150.4617	f(141-149)	/	/
MES(phospho)TEVFTKK	640.2751	1278.5567	f(141-150)	/	/
TKVIPYVRYL	417.9150	1250.7387	f(198-207)	Antimicrobial	Alvarez-Ordóñez et al., 2013

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$
[c]The calculated mass is in Da.
[d]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S8. κ-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells.

Sequence[a]	*Observed mass (m/z)*[b]	*Calculated mass*[c]	*Fragment*	*Bioactivity*[d]	*Reference*
FSDKIA	340.6721	679.3541	f(18-23)	/	/
KYIPIQY	462.7544	923.5116	f(24-30)	/	/
KYIPIQYVL	568.8299	1135.6641	f(24-32)	/	/
KYIPIQYVLS	612.3488	1222.6961	f(24-33)	/	/
SRYPSYGLN	528.7591	1055.5036	f(33-41)	/	/
RYPSYGLN	485.2353	968.4716	f(34-41)	/	/
YYQQKPVAL	555.2734	1108.5917	f(42-50)	/	/
YYQQKPVALIN	668.8605	1335.7187	f(42-52)	/	/
YYQQKPVALINN	725.8975	1449.7616	f(42-53)	/	/
QQKPVALINN	562.8214	1123.6349	f(44-53)	/	/
QKPVALINN	498.7893	995.5764	f(45-53)	/	/
QFLPYPYYAKPA	729.3794	1456.7391	f(54-65)	/	/
FLPYPYYAKPA	665.3492	1328.6805	f(55-65)	/	/
LPYPYYAKPA	591.8004	1181.6121	f(56-65)	/	/
AVRSPA	300.6625	599.3391	f(66-71)	/	/
AVRSPAQIL	477.7849	953.5658	f(66-74)	/	/
AVRSPAQILQ	541.8019	1081.6244	f(66-75)	/	/
ARHPHPHLS	351.1755	1050.5471	f(96-104)	/	/
ARHPHPHLSFM	443.8816	1328.6560	f(96-106)	/	/
EIPTIN	686.3505	685.3646	f(118-123)	/	/
TIASGEPT	775.3862	774.3759	f(124-131)	/	/
VATLEDS(phospho)PE	520.7108	1039.4111	f(143-152)	/	/
VIESPPEIN	499.2569	996.5128	f(152-160)	/	/
SPPEIN	328.6530	655.3167	f(155-160)	/	/

SPPEINTVQ	492.7466	983.4924	f(155-163)	/	/

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$.
[c]The calculated mass is in Da.
[d]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

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